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16. ABSTRACT

Following ingestion of spores of Bacillus popilliae by grubs of the European chafer, Amphimallon majalis, vegetative rods were observed within phagocytic vacuoles of midgut columnar cells prior to establishing primary infection foci in regenerative nidi areas. This resulted in increased activity of regenerative nidi and extrusion of degenerating epithelial cells frequently containing vegetative rods of B. popilliae. Circulating hemocytes adhered to the hemocoelic surface of the basement membrane and formed inflammatory capsules immediately adjacent to the areas of bacterial proliferation. Bacilli in various stages of degradation were observed in membrane-limited vacuoles of both mesenteric epithelial cells and capsular hemocytes. Despite these host defense reactions, some vegetative cells resisted degradation and were successful in traversing the epithelial, basal lamina, and capsular barriers to enter the hemolymph.

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Infection of the European Chafer, *Amphimallon majalis* by *Bacillus popilliae*: Light and Electron Microscope Observations[1]

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Following ingestion of spores of *Bacillus popilliae* by grubs of the European chafer, *Amphimallon majalis*, vegetative rods were observed within phagocytic vacuoles of midgut columnar cells prior to establishing primary infection foci in regenerative nidi areas. This resulted in increased activity of regenerative nidi and extrusion of degenerating epithelial cells frequently containing vegetative rods of *B. popilliae*. Circulating hemocytes adhered to the hemocoelic surface of the basement membrane and formed inflammatory capsules immediately adjacent to the areas of bacterial proliferation. Bacilli in various stages of degradation were observed in membrane-limited vacuoles of both mesenteric epithelial cells and capsular hemocytes. Despite these host defense reactions, some vegetative cells resisted degradation and were successful in traversing the epithelial, basal lamina, and capsular barriers to enter the hemolymph.

KEY WORDS: *Amphimallon majalis*; *Bacillus popilliae*, spores; phagocytosis; midgut columnar cells; hemocoel; hemocytes; mesenteric epithelial cells; regenerative nidi sites.

INTRODUCTION

In a previous study of the mechanism of infection of European chafer larvae by *Bacillus popilliae*, evidence was presented that, in perorally challenged grubs, spores germinated in the lumen of the alimentary canal and vegetative rods initiate a primary cycle of multiplication on the luminal side of the basement membrane prior to penetration into the hemolymph (Splittstoesser et al., 1973). The exact site of spore germination within the alimentary canal was not established. From our observations we suggested that germination probably occurred in posterior gut areas with reverse peristalsis transporting the bacteria forward to midgut invasion sites. Since the outgrowing rods are actively motile (Lüthy et al., 1970; Splittstoesser et al., 1975), they are capable of traversing the alimentary canal to susceptible epithelium and no other mechanism of movement is necessarily involved. A combination of these factors is not ruled out.

The process by which vegetative rods entered the mesenteric epithelium and penetrated to regenerative nidi sites was not observed in the earlier study. Subsequent investigations using both thick and thin sections of plastic-embedded material have revealed details of the invasion process that were not discernible with the original paraffin-embedded specimens. The results of examinations with both light and electron microscopy are the subject of this report.

MATERIALS AND METHODS

Field-collected, third-instar larvae of the European chafer were challenged by feeding potato plugs dipped in spores as described previously (Splittstoesser et al.,

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1973). At frequent time intervals, several larvae were selected for fixation. Prior to dissection, a proleg was snipped and the hemolymph was examined microscopically for the presence of vegetative rods, which would indicate penetration of the gut epithelium. Then whole midguts were dissected from grubs immersed in 3% glutaraldehyde. These were subsequently either fixed and postfixed sequentially with 3% glutaraldehyde and 1% osmium tetroxide or fixed simultaneously with both fixatives in cacodylate buffer (Hinde, 1971), dehydrated, and embedded in Spurr's plastic.

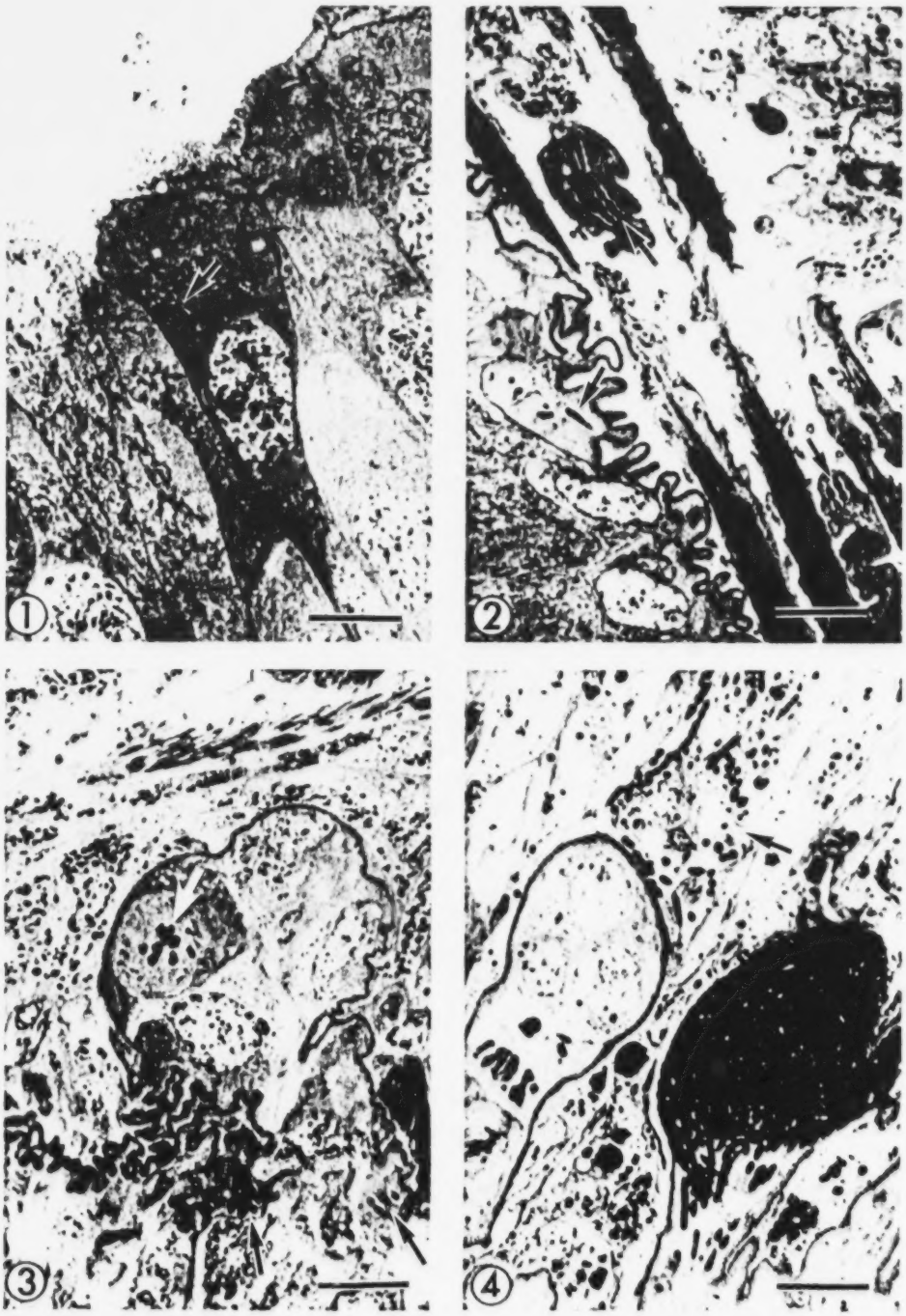
For observations under a light microscope, thick (0.6-μm) sections were cut with an ultramicrotome, placed on cleaned microscope slides, and flattened and stained by a method based on that of Roppel and Mabie (1972). A 20-mm-deep Petri dish half was used in place of their apparatus for maintaining solvent vapor over sections. The dish was fitted with a shallow layer of sponge held in place by crossed pieces of round wooden applicator sticks cut to the inner diameter of the dish. The sponge was moistened with xylene and the inverted dish was preheated prior to placing over a slide containing sections in water, as described by Roppel and Mabie (1972). An adjustable foil-covered hot plate, and a piece of 6-mm-thick glass on which the slide was placed allowed more uniform heating which was essential for reproducible staining intensities. The chafer midgut tissues did not show the differential staining described by Roppel and Mabie (1972) regardless of the proportionate amounts of each dye used. A mixture of 6:1:1 (toluidine blue:safranin O:auramine O) gave the best contrast.

For electron microscopy thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM 100B.

RESULTS AND DISCUSSION

The progress of infection at any specific fixation time varied somewhat because ingestion of inoculum was not synchronized. As with most studies of this type, the figures illustrate our conclusions based on the examination of many grubs at frequent fixation intervals. Figure 1 shows a few vegetative rods in the distal portion of an intact columnar epithelial cell from a chafer larva fixed at 12 hr postchallenge. This was the earliest fixation time at which bacteria were observed within midgut cells. The initial interaction of vegetative rod and epithelial cell was not observed. Penetration of the midgut epithelium may occur by a phagocytosis-like process such as that documented by Davidson (1973) in an investigation of the invasion of honey bee larval midgut cells by *Bacillus larvae*. Rods such as those shown in Figure 1 are apparently transported through the epithelial cell to the area adjacent to the luminal side of the basement membrane since they were never observed intercellularly. Figures 2 and 3, from specimens fixed later than 12 hr, show vegetative rods in immature replacement cells and regenerative cells near the basement membrane. These areas become the major sites of primary vegetative multiplication of *B. popilliae* in perorally challenged larvae, as evidenced by the subsequent accumulation of large masses of bacilli in regenerative nidi (Fig. 4). Intracellular rods are observed in clear membrane-limited cytoplasmic vacuoles (Fig. 5) or within dark-staining amorphous material. Many are degraded, but both damaged and normal-appearing vegetative rods may be located within a common vacuole. Davidson (1973) also noted varying stages of bacterial degradation within bee epithelial cell phagocytic vacuoles. Apparently, cell division can occur within such vacuoles as cells in fission are frequently observed. An example may be seen in Figure 6.

The presence of bacteria appears to cause increased activity of the regenerative nidi, and degenerating columnar cells frequently containing bacteria are sloughed into the lumen (Fig. 7). Although this extrusion was reported in the earlier study, it was not



FIGURES 1–4

possible to discern whether bacteria were intra- or extracellular with paraffin sections. The rate of exfoliation of mesenteric cells from infected epithelium and the mitosis of regenerative cells appears to be higher than in normal midgut. This process may be a defensive mechanism serving to confine infections to limited areas of the intestine, as suggested by Lipa and Martignoni (1960) in *Nosema*-infected midgut of the oakworm. Similar disturbed midgut epithelium and increased mitotic rate in the regenerative nidi were observed in grasshoppers challenged with *Nosema* spp. (Henry, 1967, 1969). Interestingly, these reactions are closely analagous to vertebrate reactions under inflammatory conditions. The accelerated extrusion of cells, increased mitosis of replacement cells, and subsequent appearance of numerous immature cells are associated with salmonella and shigella infections (Takeuchi, 1971). This apparently is a general response of gut mucosa to injury and is a common event under various inflammatory conditions, including bacterial infections (Takeuchi, 1971). In the chafer gut, extrusion of infected columnar epithelium is probably a defensive reaction, and the proliferation of immature cells, a compensatory one. The process of penetration of guinea pig ileal epithelium by *Salmonella typhimurium* depicted by Takeuchi (1971) shows many similarities to our observations on *B. popilliae* invasion of chafer midgut. The suggested phagocytic method of entry and the passage of envacuolated bacteria through epithelial cells followed by basement membrane penetration are similar in both systems.

The presence of bacilli in midgut tissues also evokes a defensive reaction on the part of certain circulating hemocytes. These hemocytes adhere to the hemocoelic surface of the basement membrane immediately adjacent to the bacteria and form a capsule or nodule (Splittstoesser et al., 1973). Such an aggregation of hemocytes may be seen in Figure 8. Brooks (1971) has described similar inflammatory nodules formed in microsporidian-infected lepidopterous larvae and includes in this category the "tumorous" lesions depicted by Henry (1967).

Despite these host defense processes, some vegetative rods of *B. popilliae* are able to survive and apparently penetrate the basement membrane. The actual penetration process has not been observed, but masses of bacilli are frequently seen in areas of greatly diminished or discontinuous basal lamina (Fig. 9). Bacilli on the hemocoelic side of the basal lamina within the capsule are observed both within hemocytes and in intercellular spaces. As in epithelial cells, both damaged and normal-appearing vegetative rods may be observed in vacuoles of capsular hemocytes. The ultrastructure of bacterial degradation within hemocyte and epithelial cell vacuoles and the concomitant cytological changes are the subject of a further report (Kawanishi et al., 1978).

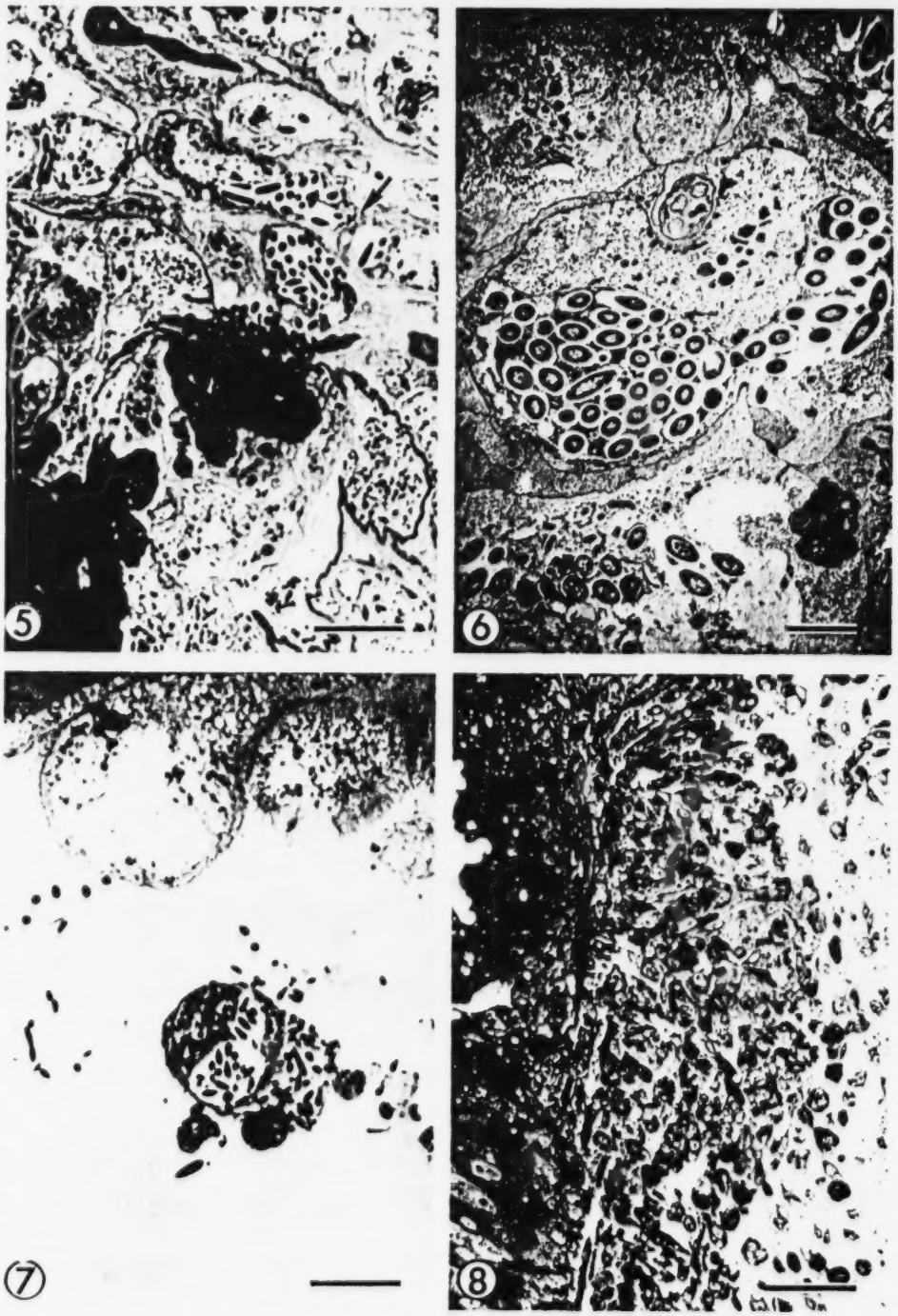
Progressing either intra- or extracellularly through the capsule, viable vegetative rods gain access to the hemolymph to continue multiplication within the body cavity of the host. Once in the hemolymph the vegetative rods are not phagocytized even though

FIG. 1. Vegetative rods (arrow) in distal portion of midgut columnar cell 12 hr postchallenge. ×1360. Bar = 10 μm.

FIG. 2. Groups of vegetative rods (arrows) in basal cells of epithelium sectioned in different planes and orientations; muscles and granule-containing hemocytes may be seen external to the gut wall; 48 hr postexposure, grub without hemolymph bacilli. ×1360. Bar = 10 μm.

FIG. 3. Vegetative rods (black arrows) near the base of a regenerative nidus; mitotic figure (white arrow) may be seen in regenerative cell; nidus is surrounded by hemocytes containing dark granules; 48 hrs postexposure. ×1360. Bar = 10 μm.

FIG. 4. Two adjacent nidi, one of which contains a dark mass of vegetative rods; in this 48-hr specimen *B. popilliae* has penetrated the basement membrane and can also be seen in the hemocytic capsule surrounding the nidi (arrow). ×1360. Bar = 10 μm.



FIGURES 5–8

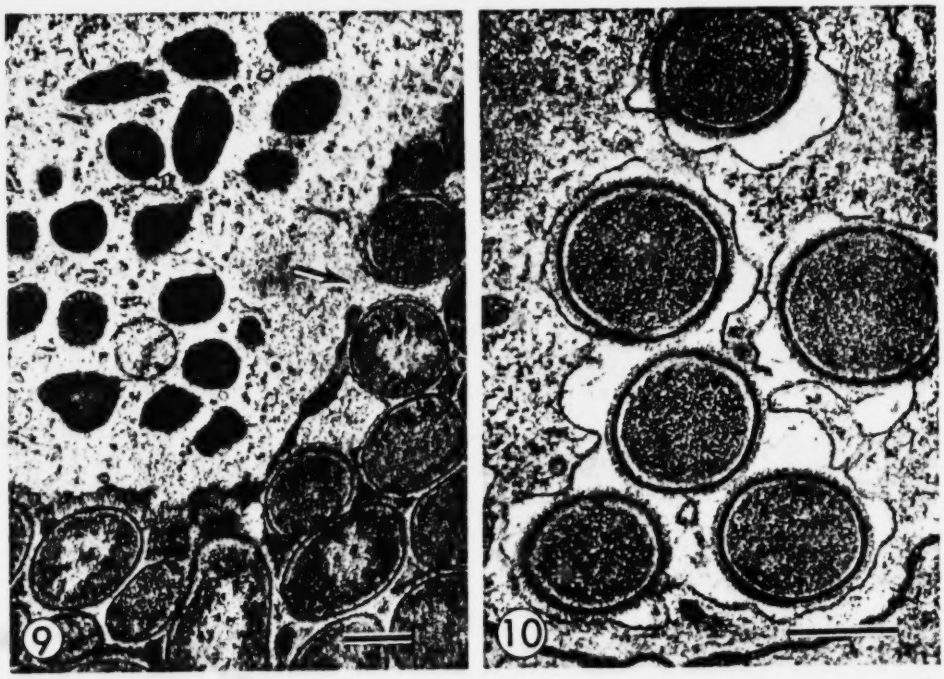


FIG. 9. Electron micrograph showing possible penetration site with bacterial cells in an area of greatly diminished basal lamina (arrow). ×19,200. Bar = 500 nm.

FIG. 10. Electron micrograph of vegetative rods showing fine fibrous surface fringe or fimbriae. ×40,290. Bar = 400 nm.

hemocytes morphologically identical to those that form the capsules are present and circulating. The reason for this phenomenon is not clear. It may be that the bacteria possess a surface structure which is not recognized as foreign by the hemocytes possibly as the result of prior intracellular growth in capsular hemocytes. Alternatively, the vegetative cells may produce an "impedin" (Glynn, 1972) while growing in the hemolymph which prevents phagocytosis by hemocytes. Dutky (1963) has described a heat-labile toxin from culture filtrates which is lethal on injection, although impedins are defined by Glynn as factors which impede host defense mechanisms without being obviously toxic themselves. The ultrastructure of vegetative rods of *B. popilliae* in vivo shows that they possess a fine fibrous external fringe or fimbriae (Fig. 10) which could be involved in preventing phagocytosis by circulating hemo-

FIG. 5. Vegetative rods in cytoplasmic vacuoles (arrow) of midgut epithelium; dark-staining areas are masses of bacteria in regenerative nidi areas. ×1360. Bar = 10 μm.

FIG. 6. Electron micrograph of vegetative rods in vacuoles near the nucleus of a midgut cell; dividing rod may be seen at right. ×5488. Bar = 2 μm.

FIG. 7. Degenerating cells containing vegetative rods sloughed into the midgut lumen. ×1360. Bar = 10 μm.

FIG. 8. Masses of hemocytes forming a capsule on hemocoelic surface of midgut adjacent to area of bacterial proliferation; infected epithelium to the left; examination showed no hemolymph bacilli. ×340. Bar = 40 μm.

cytes. Alternatively, the fimbriae might function in initial midgut epithelial cell adherence, as suggested for streptococcal fimbrae in the adherence of virulent strains to vertebrate respiratory mucosa (Ellen and Gibbons, 1972). The initial adherence might be an essential prerequisite for establishment on and penetration of enteric epithelium and the subsequent invasion processes described above. Preliminary observations suggest that in vitro grown cells of *B. popillae* lack such fimbriae, which may account for their lack of virulence per os.

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